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Central Roles for Potassium and Sucrose in Guard-Cell Osmoregulation[1]

Lawrence D. Talbott and Eduardo Zeiger*

Department of Biology, University of California, Los Angeles, California 90024

Osmoregulation in guard cells of intact, attached *Vicia faba* leaves grown under growth chamber and greenhouse conditions was studied over a daily light cycle of stomatal movements. Under both growth conditions guard cells had two distinct osmoregulatory phases. In the first (morning) phase, opening was correlated with K^+ uptake and, to a lesser extent, sucrose accumulation. In the second (afternoon) phase, in which apertures were maximal, K^+ content declined and sucrose became the dominant osmoticum. Reopening of the stomata after a CO_2-induced closure was accompanied by accumulation of either K^+ or sucrose, depending on the time of day, indicating that a single environmental signal may use multiple osmoregulatory pathways. Malate accumulation, correlated with K^+ uptake, was detected under growth chamber but not greenhouse conditions, whereas Cl^- was the main K^+ counterion in the greenhouse. These results indicate that guard-cell osmoregulation in the intact leaf depends on at least two different osmoregulatory pathways, K^+ transport and sucrose metabolism. Furthermore, the relative importance of the K^+ counterions malate and Cl^- appears to be environment-dependent.

The regulation of stomatal apertures by guard-cell osmotic potential was established well before the turn of the century (von Mohl, 1856). Early physiologists explained guard-cell osmoregulation on the basis of the starch-sugar hypothesis, which proposed interconversion between osmotically inactive starch and osmotically active sugars within the guard cell (Lloyd, 1908). This early hypothesis has been replaced with the modern concept of guard-cell osmoregulation by K^+ (Fujino, 1967; Fischer and Hsaio, 1968) and its counterions, malate and Cl^- (Allaway, 1973; Outlaw and Lowry, 1977; Van Kirk and Raschke, 1978).

The K^+ hypothesis dominates contemporary thinking in stomatal physiology. Numerous studies have documented K^+ uptake during stomatal opening (Outlaw, 1983); however, studies of *Commelina* indicated that K^+ and its counterions could not account for the required osmotica over the entire range of apertures (Macrobbie and Lettau, 1980). Carbohydrate accumulation in *Vicia* and *Commelina* has been reported in response to white light (Outlaw and Manchester, 1979; Reddy and Das, 1986). Red-light-stimulated opening of isolated *Vicia* stomata was accompanied by DCMU-sensitive Suc accumulation without detectable K^+ uptake or starch breakdown (Poffenroth et al., 1992; Talbott and Zeiger, 1993). In the same system, blue-light-stimulated opening was accompanied by transient K^+ uptake and malate synthesis, followed by Suc accumulation (Tallman and Zeiger, 1988; Poffenroth et al., 1992; Talbott and Zeiger, 1993).

Most studies of guard-cell osmoregulation have used stomata in detached epidermis or leaf pieces, incubated in artificial medium. Much has been learned with this technique, but since it is based solely on an isolated system, it carries the risk of overlooking guard-cell properties that are expressed in the intact leaf but are not apparent in detached epidermal tissue.

In the present study video image analysis was used to characterize daily courses of stomatal movements in the intact leaves of growth chamber- and greenhouse-grown *Vicia faba*. High-resolution HPLC and semiquantitative histochemistry was used to investigate the relationship between stomatal apertures and guard-cell content of Suc, K^+, malate, and Cl^-. The results indicate that both K^+ and Suc play key osmoregulatory roles in the guard cells of intact leaves and underscore some environmental effects on guard-cell osmoregulation.

MATERIALS AND METHODS

Seeds of *Vicia faba* cv Long Pod (W. Atlee Burpee, Warminster, PA) were planted in pots containing commercial potting soil (Armstrong's Garden Center, Glendora, CA). Plants were grown in a greenhouse in Los Angeles from September to May under natural sunlight, at 25 to 30°C during the day and 15 to 20°C at night. Plants were also grown in a walk-in growth chamber (PGV-36; Conviron Products, Asheville, NC) at 85% RH, 12 h of light, 650 μmol m^{-2} s^{-1} (40-W incandescent bulbs, Philips, Eindhoven, The Netherlands; F96T12/CW/VHO fluorescent bulbs, GTE Sylvania) at 25°C and 12 h of dark at 15°C. In both growth conditions plants were watered daily with an automatic watering system and fertilized (Spoonit, Morrison's Orchard Supply, Yuba City, CA) once a week.

Daily Time-Course Experiments

At each time, fully expanded, recently matured leaves from the third and fourth node of 5-week-old plants were excised and placed in a bath of ice-cold water. An abaxial epidermal peel was taken from leaves of three separate plants and immediately used for aperture determination. When K^+ or Cl^- determinations were made, a second peel was taken from each leaf and immediately used for stain-

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* Corresponding author; e-mail zeiger@lbes.medsch.ucla.edu; fax 1–310–825–9433.

ing. For organic solute determinations, duplicate samples of abaxial epidermis were prepared from two sets of four leaves. The peels were placed in ice-cold 0.1 mM $CaCl_2$ and sonicated for 28 s at 50% power on ice with a sonic homogenizer (series 4710, 300 W, 20 kHz; Cole-Parmer, Vernon Hills, IL) to disrupt mesophyll and epidermal cells. The effectiveness of the sonication procedure was assessed by fluorescence microscopy. The sonicated epidermal peels were thoroughly rinsed in distilled water and then frozen at −80°C. Total preparation time was less than 20 min, and the samples were kept cold at all times to minimize metabolite changes.

In some experiments, the stomatal apertures of growth chamber-grown plants were manipulated by artificially adjusting the ambient CO_2 level by the addition of 100% CO_2 into the fan compartment of the chamber. This location ensured good mixing of the CO_2 with the chamber air before it reached the plants. Chamber CO_2 concentration was continually monitored (EGM-1; PP Systems, Haverhill, MA).

Measurement of Stomatal Apertures

Apertures were measured from digitized video images of stomata using image-analysis hardware (MV-1, Keithley Metrabyte, Taunton, MA) and JAVA software (Jandel Scientific, Corte Madera, CA). Averages of 30 stomatal apertures were determined for each time. Total preparation time was less than 1 min, and aperture measurements were completed within 10 min of harvesting. No consistent difference between the first and last aperture measurements was observed. Empirical trials indicated that epidermal peels could be maintained on ice for 30 min without significant stomatal aperture change.

Histochemical Determinations of Inorganic Solutes

The K^+ content of guard cells was measured with sodium hexanitrocobaltate III stain (Aldrich) using freshly prepared solutions (Green et al., 1990). Staining was quantitated by determining the fraction of guard-cell area covered by stain granules (Fischer, 1971) with the image-analysis system described above.

The Cl^- content of guard cells was measured histochemically (Schnabl and Ziegler, 1977). Rinsed epidermal peels were incubated for 10 min in 1% silver lactate and 4% formaldehyde, pH 3.5. They were then incubated for 5 min in 5% acetic acid, followed by a 5-min incubation in 0.5 N NaOH containing 4% formaldehyde. The peels were rinsed in distilled water after each incubation step. Staining was quantitated by determining the average density of the guard cell on an arbitrary gray scale using the image-analysis system described above.

Although histochemical methods provide only semiquantitative information, the approach allowed us to correlate changes in guard-cell aperture and relative ion content in a rapid, well-resolved manner without interference from ions present in the rest of the epidermal tissue.

HPLC Analysis of Organic Solutes

Epidermal peels were frozen and thawed twice to rupture guard cells, and cell sap was expressed from the peels at 5°C. The peels were washed in 100 μL of cold, distilled water. The combined water wash and cell sap was passed through a 0.45-μm nylon filter, freeze-dried, and analyzed by HPLC without further handling. This procedure allows both rapid preparation and a minimum of sample handling.

An HPLC system equipped with a 2.6- × 220-mm polypore H anion-exchange chromatography column (Rainin 81–20; Alltech Associates, Deerfield, IL) was used for organic acid analysis. Samples were eluted isocratically with 0.018 M sulfuric acid at 85°C and quantitated by UV absorption at 210 nm according to calibration curves prepared with known standards.

For carbohydrate measurements, samples were analyzed with a model 2010 HPLC system (Dionex, Sunnyvale, CA) equipped with a 6.5- × 300-mm cation-exchange column (SugarPak, Waters). Samples were eluted isocratically with 50 mM Ca-EDTA buffer at 85°C and quantitated with an electrochemical detector (PADII, Dionex) after postcolumn addition of 150 mM NaOH. Dry weights of peel samples were determined and used in conjunction with empirical values for stomatal density and weight/unit surface area to normalize results on a fmol/guard-cell pair basis.

RESULTS

Daily Time Courses of Stomatal Movement

Growth chamber- and greenhouse-grown *V. faba* leaves displayed a pattern of stomatal movements that varied from day to day. In general, stomatal movements were characterized by rapid opening at the onset of light and closing late in the cycle (Figs. 1 and 2). Additionally, a midcycle depression of stomatal aperture was commonly observed in plants grown in greenhouse conditions. The specific pattern and magnitude of stomatal movements varied from day to day and was dependent on environmental conditions. Apertures under greenhouse conditions were closely correlated with incident solar radiation (data not shown). Apertures under growth chamber conditions of constant light, humidity, and temperature were closely correlated with ambient CO_2 concentration in the chamber (Talbott et al., 1996).

K^+

In five growth chamber and four greenhouse experiments, guard cells accumulated K^+ during the initial phase of opening (Fig. 1). The pattern of K^+ accumulation closely matched that of aperture increase. This correlation broke after midday, however, at which time guard-cell K^+ invariably declined to about 30 to 40% (range 13–65%) of maximum morning levels. This decrease in K^+ content never matched the pattern of aperture change and, under growth chamber conditions, occurred at a time of steady or even increasing apertures. A second peak of K^+ accumulation was observed in the afternoon, typically reaching 50 to 60%

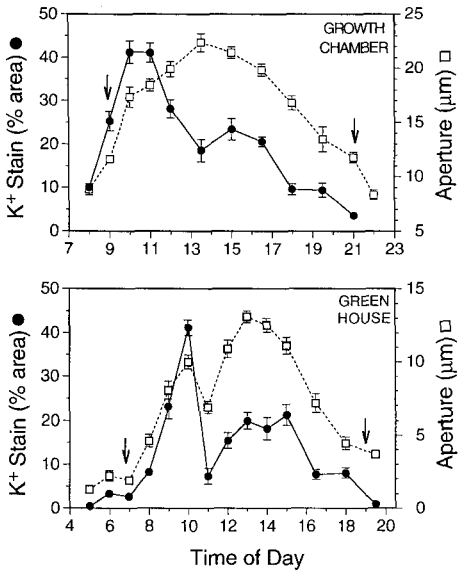

Figure 1. Aperture and K^+ content of guard cells over a daily light cycle of stomatal movements. Intact, attached leaves from growth chamber- and greenhouse-grown plants were sampled. Results are the averages ± SE of 30 measurements. Arrows show the duration of the light cycle in the growth chamber and the approximate times of sunrise and sunset in the greenhouse.

of the maximum morning level (range 35–90%). Since the maximum afternoon aperture was always equal to or greater than that of the morning, the lower afternoon K^+ levels imply that K^+ is not the sole osmoticum sustaining apertures during the later phases of opening.

Suc

Guard cells accumulated Suc in a pattern that was drastically different from that of K^+ accumulation. In five growth chamber and four greenhouse experiments, morning Suc levels were typically only 35% (range 25–40%) of afternoon levels (Fig. 2). Patterns of morning Suc and aperture changes were very poorly matched. Suc started to accumulate at faster rates at approximately the midpoint of the light cycle, and afternoon Suc levels were always more closely correlated with aperture than were K^+ levels. In the greenhouse, where a midday closure typically defined morning and afternoon aperture peaks, maximal K^+ accumulation was correlated with the morning peak, whereas maximal Suc accumulation was correlated with the afternoon peak (Figs. 1 and 2). Guard cells did not accumulate appreciable levels of Glc (Fig. 2) or other monosaccharides such as Fru, Ara, Gal, or Xyl (data not shown) under either growth condition.

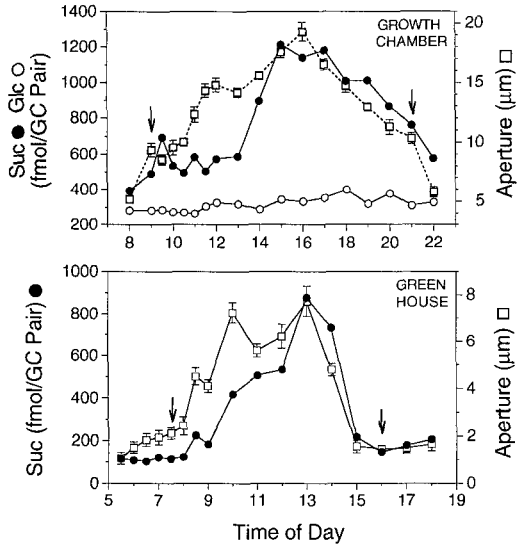

Figure 2. Aperture and Suc content of guard cells over a daily light cycle of stomatal movements. Results from growth chamber- and greenhouse-grown plants are shown as in Figure 1. Guard-cell Glc content for the growth chamber experiment is also shown. Carbohydrate points are the averages of duplicate measurements. GC, Guard cell.

K^+ Counterions

It is well established that K^+ fluxes in guard cells are balanced by malate and Cl^-. In the growth chamber (n = 5), the pattern of change in guard-cell malate content had the same biphasic character shown by K^+, with maximum malate levels seen in the morning (Fig. 3). On the other hand, there was very little change in guard-cell malate level during the light period under greenhouse conditions (n = 4), although malate accumulation was observed before dawn (Fig. 3). This accumulation coincided with a 1- to 2-μm predawn aperture increase, presumably driven by circadian rhythm (Gorton et al., 1989).

Cl^- changes exhibited the opposite pattern. In the growth chamber (n = 4), only small changes in guard-cell Cl^- were observed over the light phase of the daily course (Fig. 4). In the greenhouse (n = 3), however, substantial

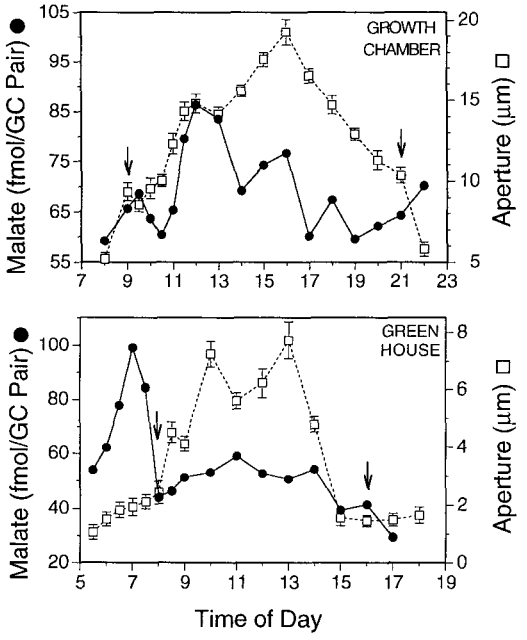

Figure 3. Aperture and malate content of guard cells over a daily light cycle of stomatal movements. Results from growth chamber- and greenhouse-grown plants are shown as in Figures 1 and 2. GC, Guard cell.

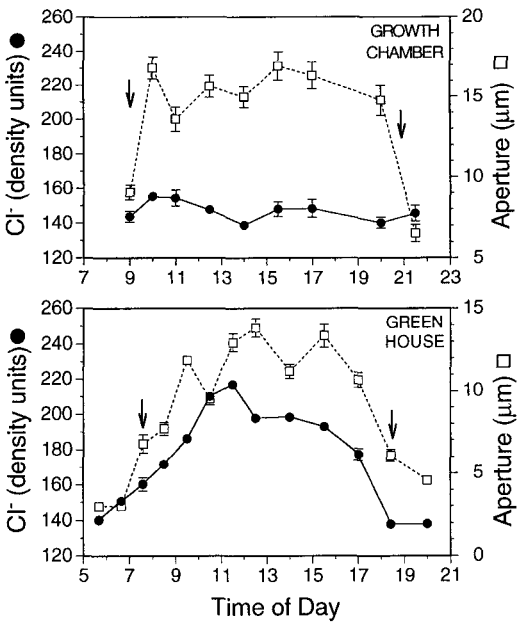

Figure 4. Aperture and chloride content of guard cells over a daily light cycle of stomatal movements. Results from growth chamber- and greenhouse-grown plants are shown as in Figure 1. Chloride results represent the averages ± SE of 30 measurements.

changes in guard-cell Cl^- content were observed (Fig. 4), and the pattern of Cl^- change closely matched the pattern of K^+ change for that day (Fig. 5).

Relationship between Stomatal Aperture and Guard-Cell Osmotica

Because of the elaborate, fine structure of aperture changes over a light cycle, a comparison of the patterns of aperture and solute changes provides a good indication of the relative osmoregulatory roles of each solute during the day. These comparisons clearly indicate that both Suc and K^+ contribute to guard-cell osmoregulation and that their relative contributions are different in the morning and the afternoon.

Further insight into the biphasic character of guard-cell osmoregulation can be gained by quantifying the relative contribution of each solute to morning and afternoon apertures (Table I). In both the growth chamber and the greenhouse, the average increase in guard-cell Suc per micrometer of opening was 3 to 4 times higher in the afternoon than in the morning. In contrast, the afternoon increase in K^+ per micrometer of opening was about one-half of that found in the morning.

Table I also indicates that the relative abundance of the K^+ counterions, malate and Cl^-, has a clear-cut environmental dependence. In the growth chamber malate contributions to opening paralleled those of K^+, but malate appeared to play no role in guard-cell osmoregulation in the greenhouse. The contribution of Cl^- to aperture paralleled that of K^+ under both growth conditions but was 3- to 4-fold higher in greenhouse plants.

Guard-Cell Solutes during CO_2-Induced Changes in Stomatal Aperture

Characterization of stomatal movements in growth chamber-grown *Vicia* leaves indicated that guard cells were very sensitive to ambient CO_2 concentration in the chamber (Talbott et al., 1996). This sensitivity made it possible to manipulate stomatal apertures by temporarily elevating the chamber CO_2 concentration, which caused rapid and reversible stomatal closure (Fig. 6a). Guard-cell K^+ content closely matched aperture changes during morning manipulations. Guard cells also lost K^+ upon closure during the afternoon manipulations, but their K^+ content remained low upon reopening (Fig. 6b). Suc, on the other hand, closely matched aperture changes during afternoon manipulations but was less closely tied to aperture during morning manipulations (Fig. 6c). The average change in K^+ content during morning manipulations was 4.4% area μm^{-1} (n = 5), which corresponds closely to that seen during the daily cycle measurements (Table I). During afternoon manipulations (n = 7), average K^+ changes during closure also agreed closely with changes seen during normal cycles (1.8 compared with 1.6% area μm^{-1}), but the K^+ increase upon reopening averaged only 0.1% area μm^{-1}. The average Suc change during afternoon manipulations was 63 fmol μm^{-1} (n = 4), which is similar to the afternoon value obtained during daily cycles (Table I). Suc changes during the morning manipulation were substantially smaller, ranging between 0 and 30 fmol μm^{-1} during both opening and closing responses.

Guard-Cell Osmoregulation during CO_2-Delayed, Afternoon Opening

The preferential use of K^+ during the first half of the light cycle and Suc during the second half raises the question of whether this biphasic osmoregulatory pattern depends on the phase of the cycle (K^+ in the morning and Suc in the afternoon) or on a requirement for a specific sequence of osmoregulatory solutes (K^+ first, then Suc). We investigated this question in experiments in which a high ambient CO_2 concentration (600 ppm) was maintained in the chamber from before the onset of the light period until 2:30 PM. The high CO_2 concentration suppressed opening

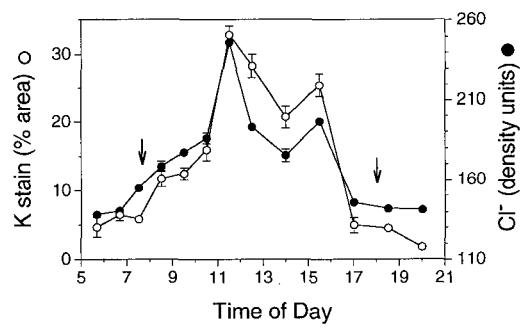

Figure 5. Comparison of guard-cell K^+ and Cl^- content over a single daily light cycle of stomatal movements in greenhouse-grown plants. Results are the averages ± SE of 30 measurements as in Figures 1 and 4.

Table I. *Guard-cell osmotica during morning and afternoon opening*

Net increase in osmoticum per guard cell pair was calculated as the increase over initial, dark levels, normalized per micrometer of opening. Morning and afternoon points were chosen at times of peak aperture/K^+ accumulation. Results are given for growth chamber (GrC) and greenhouse plants (GrH) and are the averages ± SD of three to five experiments.

Osmoticum	Net Increase per μm of Opening			
	Morning		Afternoon	
	GrC	GrH	GrC	GrH
Suc (fmol)	21 ± 4	31 ± 14	79 ± 17	97 ± 15
K^+ (% area)	3.5 ± 0.7	3.6 ± 0.7	1.6 ± 1.5	1.8 ± 0.4
Malate (fmol)	6.9 ± 2.9	−0.8 ± 0.6	3.3 ± 2.0	−0.5 ± 1
Cl^- (density units)	2.4 ± 0.3	7.9 ± 0.7	−0.5 ± 1	4.4 ± 0.8

until the time CO_2 control was released in the afternoon (Fig. 7a). Measurement of guard-cell K^+ and Suc (Fig. 7b) showed that K^+ was the dominant solute in this delayed initial opening and that the solute changes (average K^+ and Suc changes of 4.0% area μm^{-1} and 48.4 fmol μm^{-1}, respectively, $n = 2$) were typical of normal morning opening (Table I). Similar results were observed in the greenhouse when cloudy conditions in the morning delayed opening until the afternoon (data not shown).

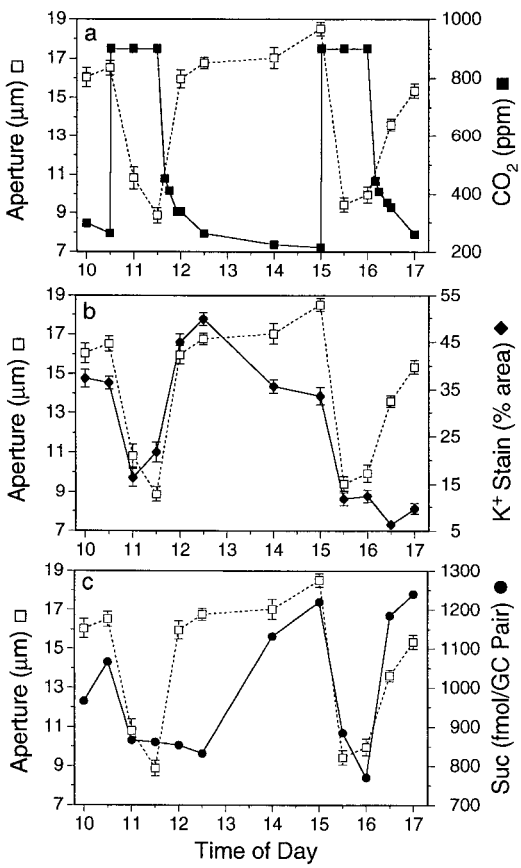

Figure 6. Osmoregulation of stomata upon artificial manipulation of aperture under growth chamber conditions. a, Aperture change in response to morning and afternoon pulses of elevated ambient CO_2 concentration applied at 11 AM and 3 PM. K^+ (b) and Suc (c) content of guard cells during morning and afternoon aperture changes. ppm, Parts per million. GC, Guard cell.

DISCUSSION

The observation of K^+ efflux prior to actual stomatal closure is consistent with data from experiments with isolated stomata in epidermal peels, which showed a K^+-dependent opening phase under both white and blue light, followed by K^+ loss and increases in Suc content (Rodgers et al., 1980; Tallman and Zeiger, 1988; Talbott and Zeiger, 1993). This indicates that in the intact leaf the control of K^+ efflux would not necessarily regulate stomatal closure, as inferred from studies with isolated guard cells and their protoplasts (Schroeder et al., 1993). It seems, rather, that closure during a normal daily cycle is more tightly coupled to a decrease in guard-cell Suc.

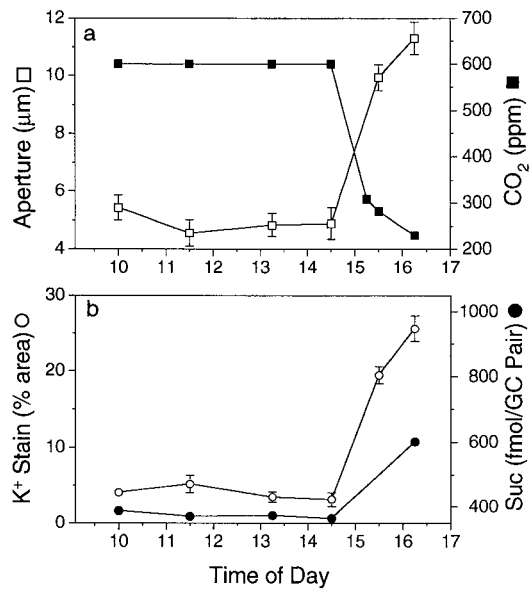

Figure 7. Apertures and guard cell osmoregulation during afternoon opening in the growth chamber under conditions in which the morning opening was suppressed by elevated ambient CO_2 concentration. a, Aperture and chamber CO_2 concentration; b, guard-cell Suc and K^+ content. ppm, Parts per million; GC, guard cell.

An estimate of the absolute contribution of Suc to aperture can be obtained by calculating the expected change in aperture resulting from the observed accumulation of Suc. Using the data in Figure 2 for greenhouse plants and published estimates of guard-cell volume (4 pL) and osmotic potential change per unit aperture for unsonicated greenhouse *Vicia* epidermal peels (0.05–0.1 MPa/μm), the peak afternoon Suc accumulation of 400 fmol/guard cell can be calculated to produce a 100 mM (0.26 MPa) increase in solute concentration, or a 2.6- to 5.2-μm increase in aperture (Poffenroth et al., 1992). Similar calculations yield an estimated 0.8- to 1.7-μm change in aperture resulting from morning Suc accumulation. Thus, observed changes in Suc concentration could account for 14 to 28% and 37 to 74% of observed morning and afternoon aperture changes, respectively, exclusive of any other osmoticum.

The role of Suc as a cytosolic osmoticum has been a classic concept in stomatal physiology (Raschke, 1975; Outlaw, 1983) but does not explain the shift from K^+ to Suc in the afternoon phase of the daily cycle. The experiments in which stomatal opening was suppressed until the afternoon showed that this opening was supported by K^+ uptake—evidence against a circadian rhythm-driven control of osmotic type. Since the elevated CO_2 concentration used to suppress opening supports substantial morning photosynthetic rates (data not shown), these experiments also indicate that the preferential use of Suc in the second phase of undisturbed time courses is not causally related to higher afternoon apoplastic Suc concentrations resulting from mesophyll photosynthesis (Pearson, 1973; Basu and Minhas, 1991).

The close association between initial opening and K^+ uptake could mean that K^+ accumulation is used primarily for rapid opening, whereas Suc is used for turgor maintenance and for the regulation of stomatal closure. However, the CO_2 pulse experiments show that rapid afternoon opening is possible using Suc.

Taken together, the daily cycles and CO_2 experiments suggest an osmoregulatory sequence that requires K^+ to be used first. K^+ accumulation to some critical level may trigger a shift toward Suc-based osmoregulation and initiate a metabolic state in which the guard cell has a reduced capacity to use K^+ until the sequence is reset by a period of closure in the dark. A K^+-first requirement would be consistent with the observed slow, suboptimal, Suc-dependent opening under red light, which occurs without any detectable K^+ uptake (Tallman and Zeiger, 1988; Talbott and Zeiger, 1993). Functionally, osmoregulatory pathways producing Suc could be more tightly coupled to photosynthetic rate and thus represent a mechanism whereby stomatal apertures may be more finely tuned to the rate of carbon fixation during midday and the early afternoon.

Implications for Environmental Sensing

The current understanding of the regulation of stomatal movements emphasizes a sensory transduction sequence whereby a specific environmental signal would directly modulate the activity of a specific metabolic pathway. The large, rapid changes in guard-cell solute elicited by the CO_2 pulses indicate that the CO_2 signal is not solute specific and can modulate stomatal apertures via different osmoregulatory pathways. This finding suggests a mechanism by which environmental signals are transduced into appropriate apertures through a solute-independent guard-cell parameter such as turgor. Prevailing cellular conditions then determine which osmoregulatory pathway(s) is activated to generate the required solutes. Such a mechanism is different from aperture modulation through direct regulation of specific osmoregulatory pathway enzymes, such as light stimulation of a redox proton pump (Raghavendra, 1990) or NADP-malate dehydrogenase (Gotow et al., 1985).

The differences in guard-cell malate and Cl^- content in the growth chamber and greenhouse, despite similar patterns of K^+ accumulation, point to substantial plasticity in the use of K^+ counterions. Blue light is known to stimulate malate biosynthesis in guard cells (Ogawa et al., 1978). Blue-light enrichment of growth chamber light sources (Lu et al., 1993) could explain the enhanced use of malate under these conditions. However, the levels of malate found in the stomata of these intact leaves were substantially less than those found previously in isolated stomata of epidermal peels (Talbott and Zeiger, 1993). Maximum malate accumulation in isolated guard cells was approximately 43 compared with 6.9 fmol/μm in the intact system.

Most of the accumulated evidence for a primary role of malate in guard-cell osmoregulation has been inferred from experiments with isolated guard cells incubated in artificial medium. The present findings show that the stomata of intact growth chamber-grown leaves accumulate substantially less malate, whereas stomata of greenhouse-grown leaves, which most closely approximate those in a natural environment, do not show any substantial malate accumulation over the light cycle. This indicates that in the intact system other counterions, including but not limited to Cl^-, may be used to balance K^+.

Malate does accumulate in the stomata of greenhouse-grown leaves in conjunction with a small (1–2 μm) predawn opening. Although K^+ accumulation during this opening is far below maximal, malate is also involved in other metabolic functions, such as respiration, which may be active during this period.

Origin of Suc

The source of accumulated Suc in guard cells remains to be determined. In contrast to the extensively studied osmoregulatory pathway of K^+ and its counterions (Assmann, 1993), much remains to be learned about Suc metabolism and transport in guard cells. Work with isolated guard cells has characterized two osmoregulatory pathways involved in Suc biosynthesis: photosynthetic carbon fixation and starch degradation (Gotow et al., 1988; Tallman and Zeiger, 1988; Poffenroth et al., 1992; Talbott and Zeiger, 1993). Import of apoplastic Suc is a third possible source in the intact leaf (Reddy and Das, 1986). Available evidence suggests that all three Suc

sources might prove to be functionally relevant in some physiological conditions.

The observation of important roles for both K^+ and Suc as guard-cell solutes unifies the classic starch-sugar hypothesis with the contemporary paradigm of osmoregulation by K^+ and its counterions. Understanding the regulation of the alternative osmoregulatory pathways and their specific functional roles emerges as a key issue in guard-cell biology.

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